INHIBITING NEOPLASTIC CELLS UTILIZING THE RIBONUCLEOTIDE REDUCTASE UTR

This application claims benefit under 35 USC §119(e) of United States Provisional Application Serial Number 60/021,152, filed July 1, 1996.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The field of this invention relates to methods of controlling the tumorigenicity and/or metastasis of neoplastic cells. Specifically it relates to the use of the untranslated regions (UTR) of mRNA for housekeeping genes to inhibit neoplastic cells and more specifically the R1 and R2 components of ribonucleotide reductase UTR.

BACKGROUND ART

The first unique step leading to DNA synthesis is the conversion of ribonucleotides to their corresponding deoxyribonucleotides, a reaction that is catalyzed in a cell cycle specific manner by the housekeeping gene ribonucleotide reductase [Lewis et al., 1978; Reichard, 1993; Wright, 1989a; Wright et al., 1990a; Stubbe, 1989]. The mammalian enzyme is composed of two dissimilar dimeric protein components often called R1 and R2, which are encoded by two different genes located on different chromosomes [Björklund et al., 1993; Tonin et al., 1987]. Mammalian protein R1 is a homodimeric structure, with a molecular weight of about 170 kDa, and has substrate sites and allosteric effector sites that control enzyme activity and substrate specificity [Wright, 1989; Thelander et al., 1980; Caras et al., 1985; Wright et al., 1990a]. Protein R2 is a homodimer, with a molecular weight of 88 kDa, and

forms two equivalent dinuclear iron centers that stabilizes a tyrosyl free radical required for catalysis [Wright et al., 1990a; Thelander et al., 1985; McClarty et al., 1990]. R1 and R2 proteins interact at their C-terminal ends to form an active holoenzyme [Reichard, 1993; Wright et al., 1990a; Davis et al., 1994].

R1 and R2 are differentially regulated during the cell cycle. There is an S-phase correlated increase in the R2 protein resulting from its *de novo* synthesis [Lewis et al., 1978; Mann et al, 1988]. The activity of ribonucleotide reductase, and therefore DNA synthesis and cell proliferation, is controlled in proliferating cells during the cell cycle by the synthesis and degradation of the R2 component [Eriksson et al., 1984]. The rate-limiting R2 component is a phosphoprotein capable of being phosphorylated by the CDC2 and CDK2 protein kinase mediators of cell cycle progression [Chan et al., 1993], and contains non-heme iron that stabilizes an unique tyrosyl free radical required for enzyme activity [Reichard, 1993; McClarty et al., 1990].

The levels of the R1 protein do not appear to change substantially during the cell cycle of proliferating cells and can be detected throughout the cell cycle. Synthesis of R1 mRNA, like R2 mRNA appears to occur mainly during S phase [Eriksson et al., 1984; Choy et al., 1988; Mann et al., 1988]. The broader distribution of the R1 protein during the cell cycle is attributed to its longer half life as compared to the R2 protein [Choy et al., 1988; Mann et al., 1988].

Regulation of ribonucleotide reductase, and particularly the R2 component, is markedly altered in malignant cells exposed to tumor promoters or to the growth factor TGF-β [Amara, et al., 1994; Chen et al., 1993; Amara et al., 1995b; Hurta and Wright, 1995; Hurta et al., 1991]. Higher levels of enzyme activity have been observed in cultured malignant cells when compared to nonmalignant

cells [Weber, 1983; Takeda and Weber, 1981; Wright et al., 1989a], and increased levels of R2 protein and R2 mRNA have been found in pre-malignant and malignant tissues as compared to normal control tissue samples [Saeki et al., 1995; Jensen et al., 1994]. Regulation of ribonucleotide reductase, and in particular the R2 component, is significantly elevated in transformed cells exposed to tumor promoters, or to transforming growth factor β in growth factor mediated mechanisms of tumor progression [Amara et al., 1996; Chen et al., 1993; Amara et al, 1995b]. These studies are in tumor cells obtained from rodent and human tissues [Weber, 1983; Wright et al., 1989a; Saeki, et al., 1995; Jenson et al, 1994], and in cultured cells selected for resistance to anti-tumor agents such as hydroxyurea [Lewis et al., 1978; Wright et al., 1989b]. These observations suggest that ribonucleotide reductase may be involved in mechanisms controlling malignant progression.

Chemotherapeutic compounds like hydroxyurea inhibit ribonucleotide reductase activity by destabilizing the iron center of the R2 protein causing the destruction of the tyrosyl free radical [McClarty et al., 1990], and preventing cells from progressing through S-phase of the cell cycle [Ashihara and Baserga, 1979]. In addition to cell cycle control, ribonucleotide reductase can be regulated by an S-phase independent mechanism that is important for DNA repair. Ribonucleotide reductase activity can be induced outside the S phase by DNA cross-linking agents such as chlorambucil, and by UV irradiation indicating a role for the enzyme in the DNA repair process [Hurta and Wright, 1992; Filatov, et al., 1996].

Recent studies have shown that ribonucleotide reductase activity is quickly elevated in the presence of tumor promoters like 12-0-tetradecanoylphorbol-13-acetate [Amara et al., 1994; Chen et al., 1993]. This process is mediated at least in part, through increases in the half-lives of R1 and R2 mRNAs, which parallels the decreased

interactions of two proteins, R1BP and R2BP, with *cis*-element sequences in the 3′ untranslated regions (3′ UTRs) of the R1 and R2 messages [Amara et al., 1994; Chen et al., 1993; Chen et al., 1994a; Chen et al., 1994b]. Alterations in this *cis-trans* reaction can play a role in determining sensitivity to chemotherapeutic agents that target ribonucleotide reductase [Amara et al., 1995a].

Exposure of transformed fibroblasts to TGF-β_1 can increase the half-life of the R2 message, a process that is mediated through a *cis-trans* interaction within the R2 mRNA 3′ UTR [Amara et al., 1995b; Hurta and Wright, 1995]. Other studies have demonstrated that the non-coding regions of mRNAs can control important biological properties of cells, such as the expression of bFGF in *Xenopus* oocytes [Kimelman and Kirschner, 1989], the timing of developmental events of *Caenorhabditis elegans* [Lee et al., 1993], the expression of α1 (I) collagen in chick embryo chondrocytes [Farrell and Lukens, 1995], and the suppression of tumorigenicity of rhabdomyosarcomas by RNA from the 3′ UTR of the non-housekeeping gene α-tropomyosin [Rastinejad et al., 1993].

PCT patent application WO 94/21661 further discusses the use of UTRs of cell structural proteins to regulate cell division and/or cell differentiation and provides a discussion of how exogenous UTR may affect cell regulation and is incorporated herein in its entirety by reference. The application specifically indicates that the UTRs should not be those from housekeeping genes and defines housekeeping genes as those, among others, associated with DNA replication, transcription and RNA translation.

Housekeeping genes are in general referred to as genes/functions/activities that are required by most cells and critically linked to general cell metabolism as opposed to "luxury" genes/functions/activities that are used by specialized cells and tissues of a multicellular organism [see in general "Molecular Biology of the Gene", Alberts et al., Garland Publishing Inc., New York, 1983; "Explorations

in Developmental Biology" (Eds C. Fulton and A. O. Klein) Vail-Ballou Press, Inc. 1976; "Principles of Genetics" Herskowitz, The Macmillian Company, New York, 1973]. Examples of housekeeping genes/proteins include ribonuclease reductase, ornithine decarboxylase (ODC), dihydrofolate reductase, CAD (multifunctional gene that encodes carbamoyl phosphate synthetase, aspartate transcarbamylase and dihydrooortase), and glyceraldehyde phosphate dehydrogenase (see also Table 3). Luxury genes/proteins, which include α-tropomyosin, hemoglobin, lens crystallins and the like, are genes or proteins required to determine specialized cellular functions.

The regulation of mRNA turnover is an essential step in controlling message abundance and therefore gene expression in mammalian cells. Message degradation or stability plays a critical role in cell proliferation or cellular differentiation, and is crucial in mechanisms that maintain normal biological functions of individual cells and tissues. Aberrant mRNA turnover usually leads to altered levels of proteins, which can dramatically modify cellular properties. For example, oncogene or growth factor overexpression is often associated with abnormal cell proliferation and malignant transformation. Since message turnover is an important component of gene regulation, it is not surprising to find that message stability characteristics of key growth regulatory genes are tightly controlled. Several excellent reviews are available which describe in detail mechanisms of gene expression that are regulated at the mRNA level [Ross, 1995; Hake and Richer, 1997].

Messenger RNA is composed of distinct domains that either encode proteins or carry specific regulatory regions that control gene expression posttranscriptionally. Structurally there are three distinct regions of an mRNA molecule, the 5′ end including the cap (5′-GpppG--), the coding region, and the 3′ end including the polyadenylated tail. The structural elements of mRNA are known to play

integral roles in mechanisms regulating translation and mRNA stability, which in turn directly affect translation efficiency and the turnover rate of the message, and therefore the amount of a specific protein that is synthesized.

The 5′ end of an mRNA molecule contains a sequence that is not translated into protein and therefore is known as the 5′ untranslated region (UTR), and contains the mRNA cap which confers nuclease resistance properties. There is a great deal of evidence showing that the 5′ end of a message is critically involved in regulating translation initiation [Ross, 1995; Hake and Richer, 1997]. Alterations in translation regulation not only directly affects the amount of a protein that is eventually synthesized, but it can also significantly modify the stability characteristics of the message and therefore modify protein levels by this mechanism as well. For example, some viruses are capable of modifying the binding of regulatory proteins to the 5′ UTR including the cap region, and through this process control host versus virus gene expression. The 5′ UTR of a message can be relatively short or can be several hundred nucleotides in length.

There is also a region of varying length following the coding sequence that is not translated into protein, and this 3′ UTR which may be many hundreds of nucleotides in length, appears to play a dominant role in determining message stability characteristics. There are now many examples of unique *cis*-elements in this part of the message that bind to *trans*-acting proteins to control mRNA turnover rates [Ross, 1995; Hake and Richer, 1997].

In addition, most mRNAs have a polyadenylated (poly (A)) tail at the 3′ end, which can serve several functions important to translation efficiency and message turnover characteristics. For example the poly (A) tail protects the message from degradation in some systems, and it has been demonstrated that deadenylation may be the first step in message degradation. The mere presence of a poly (A)

tail is not necessarily sufficient for protection, instead the poly (A) tail should be a minimum length, for example 20 to 30 nucleotides long, to provide protection from nuclease action. When the number of residues is changed experimentally, the rate of degradation can be increased or decreased by the absence or presence of a specific number of residues. Several proteins are involved in this regulation including a poly (A) binding protein, and it has been suggested that the poly (A) tail blocks the assembly of an exonuclease involved in RNA degradation [Sachs, 1993; Ford et al., 1997].

Besides the interactions between *cis*-elements with precise nucleotide sequences and *trans*-acting proteins, secondary structural conformations such as stem-loops and hairpin structures also serve regulatory functions in the untranslated regions (UTRs) of mRNAs. For example, it has been shown in some cases, that it is possible to transfer sequences containing interesting structural features from the UTR from one mRNA to another and alter the stability characteristics of the recipient mRNA. Certainly, stem-loop structures play important roles in message regulation of histone mRNA [Marzluff and Pandey, 1988], or ferritin and transferrin receptor mRNA regulation [Klausner and Hartford, 1989]. Histone mRNAs are cell cycle regulated and lack a poly (A) tail, but structural information in the 3' UTR including a 6 base pair stem and 4 base loop motif found in all histone mRNAs, play crucial roles in controlling the rates of translation and degradation. In general, secondary structural features are important because they influence the binding of regulatory proteins that directly or indirectly affect interactions between the mRNA and nucleases and/or because they may act directly as favored recognition sites for particular nuclease activities or as inhibitors of nuclease action.

The genetic changes underlying cancer conversion and progression are accompanied by a decrease in genomic stability of cells [Cifone and Fidler, 1981; Wolman, 1983;

Rowley, 1990; Huang et al., 1995a], which leads to heterogeneity of tumor cell populations, alterations in response to chemotherapy, and increased malignant potential. The multitude of changes that are observed during malignant transformation and are most pronounced at advanced stages of the disease, are at least in part due to changes in genome/message stability, as manifested for example by an increased potential for DNA amplification [Rowley, 1990; Wright et al., 1990b; Tlsty, 1990]. Normal diploid cells rarely amplify their DNA, but amplification of oncogenes and genes determining drug resistance is often observed in tumor cell populations, and this is one of the most impressive characteristics that distinguishes normal cells from tumor cells [Wright et al., 1990b; Tlsty, 1990]. The expression of several genes that are known to play fundamental roles in malignant progression are strictly regulated at the posttranscriptional level through mechanisms that control message stability characteristics. Clearly, mechanisms that lead to genomic/message destabilization are important in cancer transformation and progression, and methods are needed for reversing or controlling genomic destabilization which can be utilized in treating cancer.

SUMMARY OF THE INVENTION

The present invention relates to oligonucleotides having the sequence of an untranslated region (UTR) of mRNA from a housekeeping gene and which can modulate the tumorigenicity of neoplastic cells. The present invention further provides pharmaceutical compositions and methods of inhibiting the growth of neoplastic/malignant cells in a mammal with oligonucleotides having sequences of untranslated regions of the mRNA of housekeeping genes. In a preferred embodiment the UTR is the 3'-UTR and the housekeeping gene is ribonucleotide reductase and its regulatory sequences.

According to the present invention a synthetic oligonucleotide comprising at least seven nucleotides or nucleotide analogues having a sequence corresponding to a consecutive segment of an untranslated 3' region (3'-UTR) of mRNA of a housekeeping gene is disclosed. In a preferred embodiment the oligonucleotide has a sequence selected from the entire 3'-UTR of the mRNA for the R1 or R2 component of ribonucleotide reductase or segments thereof substantially free of the coding sequence of ribonucleotide reductase protein R1 or R2 respectively. Alternatively the antisense sequences thereof or ribozymes comprising a sequence complementary to at least a portion of the R1 or R2 3'-UTR.

The present invention provides a pharmaceutical composition for inhibiting tumorigenicity of a neoplastic cell in a mammal. The pharmaceutical composition consists of a synthetic oligonucleotide comprising at least seven nucleotides or nucleotide analogues having a sequence corresponding to a consecutive segment of an untranslated 3' region (3'-UTR) of mRNA of a housekeeping gene and a pharmaceutically physiologically acceptable carrier or diluent. In a preferred embodiment the composition includes an effective amount of an active ingredient selected from oligonucleotides having a sequence corresponding to the entire 3'-UTR of the mRNA for the R1 or R2 component or segments thereof substantially free of the coding sequence of ribonucleotide reductase protein R1 or R2 respectively or the antisense sequences thereof or ribozymes comprising a sequence complementary to a portion of the 3'-UTR in a pharmaceutically physiologically acceptable carrier or diluent.

According to the present invention, a method of inhibiting the growth of a neoplastic cell in a mammal by utilizing the UTR of housekeeping genes mRNA is provided. The method includes contacting the neoplastic cells with a growth inhibiting amount of at least one oligonucleotide having a sequence of at least seven consecutive nucleotides

or nucleotide analogues of a 3′ untranslated region (3′-UTR) of mRNA of a housekeeping gene of the mammal substantially free of the coding sequence of the housekeeping gene. In a preferred embodiment, the method includes methods to control undifferentiated cell growth and/or metastatic potential in premalignant or malignant cells wherein the mRNA UTRs of the R1 and R2 components of ribonucleotide reductase are utilized.

The present invention also provides for antibodies directed against the oligonucleotides selected from the group having a sequence corresponding to the entire 3′-UTR of the mRNA for the R1 or R2 component or segments thereof substantially free of the coding sequence of ribonucleotide reductase protein R1 or R2 respectively.

The present invention provides methods for identifying a substance that modulates the tumorigenic properties of a cell and for screening for an agonist or antagonist of the interaction of an oligonucleotide with a substance which binds to the oligonucleotide.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1 is a photograph of a gel showing the expression of recombinant 3′ UTRs in vector-transfected RMP-6 cells (Example 1). Total cellular RNA pretreated with or without DNase-free RNase A was used for the reverse transcriptase-PCR. The 5′ and 3′ primers were directed towards the UTRs and the vector, respectively. RMP-VC (Lanes 2, 7, 12), RMPM1U (Lanes 3,4, 8,9), RMPM2U (Lanes 5,6) and RMPC 0.8 (Lanes 13,14) were derived from RMP-6 cells after transfection with the vector control, the vector containing the R1 3′ UTR, the vector containing the R2 3′ UTR , or a chlamydial sequence by calcium phosphate

precipitation, respectively. eRMP-VC (Lane 12), eRMPM1U (Lanes 5,6) and eRMPM2U (Lanes 10,11) were obtained by electroporation of the plasmids into RMP-6 cells. The left lane (Lane 1) shows the migration of 100 bp ladder marker (Pharmacia), and the lowest band is 100 bp.

FIGURE 2 is a graph showing the growth of subcutaneous tumors in syngeneic mice (Example 1). The data for each point ± SE represents the results obtained for five mice. The latency periods for RMP-VC (●), RMPM1U (■) and RMPM2U (▲) tumor cells were 8, 9 and 10 days following injection. Examination of the slopes of the curves indicated that the tumor growth rate of RMP-VC cells was significantly greater than the rate for RMPM1U ($p < 0.01$) or RMPM2U ($p < 0.005$) cells, respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention relates to oligonucleotides having the sequence of an untranslated region (UTR) of mRNA from a housekeeping gene and which can modulate the tumorigenicity of neoplastic cells. The present invention further provides pharmaceutical compositions and methods of inhibiting the growth of neoplastic/malignant cells in a mammal with oligonucleotides having sequences of untranslated regions of the mRNA of housekeeping genes. In a preferred embodiment the UTR is the 3'-UTR and the housekeeping gene is the ribonucleotide reductase genes and its regulatory sequences.

By inhibition of growth is meant that the tumor cell returns to a differentiated normal growth pattern and/or is killed and/or surviving cells are no longer tumorigenic (undifferentiated cell division or growth; tumor forming) or metastatic.

The term "housekeeping gene" as used herein is a gene that is generally expressed in most cell types during the cell cycle and is required for cell survival as discussed herein above. The housekeeping gene may be any

housekeeping gene. The housekeeping gene can be a gene encoding a protein regulating DNA synthesis and repair and can be involved in purine and pyrimidine synthesis or other necessary functions. Examples of other housekeeping genes are shown in Table 3. A preferred housekeeping gene of the present invention is ribonucleotide reductase which is involved in the conversion of ribonucleotides to their corresponding deoxyribonucleotides.

The present invention provides a synthetic oligonucleotide comprising at least seven nucleotides or nucleotide analogues and can be formed from ribonucleotides or deoxyribonucleotides. The oligonucleotides have a sequence corresponding to the entire untranslated 3' region (3'-UTR) of mRNA of a housekeeping gene or to a consecutive nucleotide sequence segment of at least seven nucleotides corresponding to the region.

The segment is selected such that the sequence exhibits the least likelihood of showing dimer formation, self-complementary interactions and binding potential to the UTR sequence. In other words reduced dimer formation, reduced self-complementary interactions and reduced binding potential to the UTR sequence as determined by using the computer modeling program OLIGO Primer Analysis Soft ware, Version 3.4 (distributed by National Biosciences). The program allows the determination of a qualitative estimation of these three parameters and ranks them as "no potential" or "some potential" or "essentially complete potential". Segments were generally selected that had estimates of no potential in all three parameters. However, several segments as shown in Table 5 had parameters that were in the "some potential" category and were still effective having a reduced (some) potential. A balance of the parameters is used in the selection.

In an embodiment the synthetic oligonucleotide of the housekeeping gene encodes ribonucleotide reductase dimeric protein components designated R1 and R2. As described herein above, two genes (R1, sometimes called M1, and R2,

sometimes called M2) on separate chromosomes are involved in the production of the protein. As discussed herein, when referring to ribonucleotide reductase gene, both genes are implied unless specifically noted.

The synthetic oligonucleotides of this embodiment of the present invention can have a sequence corresponding to the entire 3'-UTR of the mRNA for the R1 component or segment thereof substantially free of the coding sequence of ribonucleotide reductase protein R1. A noninclusive list of R1 oligonucleotide segments is found in Table 4 (SEQ ID Nos:44-49). In a preferred embodiment the oligonucleotide segment has a sequence set forth in SEQ ID No:45.

The synthetic oligonucleotides of the present invention can have a sequence corresponding to the entire 3'-UTR of the mRNA for the R2 component or segment thereof substantially free of the coding sequence of ribonucleotide reductase protein R2. A noninclusive list of the oligonucleotide segments is found in Table 5 (SEQ ID Nos:6-43). In a preferred embodiment the oligonucleotide segments have a sequence set forth in SEQ ID Nos:6-12.

The segment is selected such that the sequence exhibits the least likelihood of showing dimer formation, self-complementary interactions and binding potential to the respective R1 or R2 3'-UTR mRNAs (as estimated in columns D, H and A of tables 4 and 5) and as described herein above. It should be noted that a synthetic oligonucleotide of the present invention can comprise at least two sequences of a consecutive segment of an untranslated 3' region (3'-UTR) of the mRNA of a housekeeping gene linked together.

Modifications or analogues of nucleotides can be introduced to improve the therapeutic properties of the oligonucleotides. Improved properties include increased nuclease resistance and/or increased ability to permeate cell membranes.

Nuclease resistance, where needed, is provided by any method known in the art that does not substantially interfere with biological activity of the UTRs and segments thereof, antisense oligodeoxynucleotides directed to the UTR sequence, ribozymes as needed for the method of use and delivery [Iyer et al., 1990; Radhakrishnan, et al., 1990; Eckstein, 1985; Spitzer and Eckstein, 1988; Woolf et al., 1990; Shaw et al., 1991]. Modifications that can be made to oligonucleotides in order to enhance nuclease resistance include modifying the phosphorous or oxygen heteroatom in the phosphate backbone. These include preparing methyl phosphonates, phosphorothioates, phosphorodithioates and morpholino oligomers. In one embodiment it is provided by having phosphorothioate bonds linking between the four to six 3'-terminus nucleotide bases. Alternatively, phosphorothioate bonds link all the nucleotide bases. Other modifications known in the art may be used where the biological activity is retained, but the stability to nucleases is substantially increased.

The present invention also includes all analogues of, or modifications to, an oligonucleotide of the invention that does not substantially affect the function of the oligonucleotide. The nucleotides can be selected from naturally occurring or synthetically modified bases. Naturally occurring bases include adenine, guanine, cytosine, thymine and uracil. Modified bases of the oligonucleotides include xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thioalkyl guanines, 8-hydroxyl guanine and other substituted guanines, other aza and deaza adenines, other aza and deaza guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

In addition, analogues of nucleotides can be prepared wherein the structure of the nucleotide is fundamentally altered and that are better suited as therapeutic or experimental reagents. An example of a nucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in DNA (or RNA) is replaced with a polyamide backbone which is similar to that found in peptides. PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. Further, PNAs have been shown to bind stronger to a complementary DNA sequence than a DNA molecule. This observation is attributed to the lack of charge repulsion between the PNA strand and the DNA strand. Other modifications that can be made to oligonucleotides include polymer backbones, cyclic backbones, or acyclic backbones.

Short synthetic antisense (AS) nucleotide sequences designed to hybridize with specific sequences within a targeted mRNA have been shown to suppress gene function. Therefore, the present invention also allows for the use of the antisense oligonucleotide sequence of the UTR of a housekeeping gene. In an embodiment the R1 UTR or R2 UTR is used. The antisense sequence may be for the entire UTR sequence or antisense nucleotides may be targeted to segments of the UTR sequence. The antisense nucleotides are made and delivered as discussed herein below. The antisense oligonucleotides may be used in combination with the UTR oligonucleotide sequences.

Many reviews have covered the main aspects of antisense (AS) technology and its therapeutic potential [Wright and Anazodo, 1995]. There are reviews on the chemical [Crooke, 1995], cellular [Wagner, 1994] and therapeutic [Hanania, *et al*, 1995; Scanlon, *et al*, 1995; Gewirtz, 1993] aspects of this technology. Ample information has accumulated about the *in vitro* use of AS nucleotide sequences in cultured primary cells and cell lines as well as for *in vivo* administration of such

nucleotide sequences for suppressing specific processes and changing body functions in a transient manner. Further, enough experience is now available *in vitro* and *in vivo* in animal models and human clinical trials to predict human efficacy.

Antisense intervention in the expression of specific genes can be achieved by the use of synthetic AS oligonucleotide sequences [for recent reports see Lefebvre-d'Hellencourt *et al*, 1995; Agrawal, 1996; Lev-Lehman *et al*, 1997]. AS oligonucleotide sequences may be short sequences of DNA, typically 15-30 mer but may be as small as 7 mer [Wagner et al, 1996], designed to complement a target mRNA of interest and form an RNA:AS duplex. This duplex formation can prevent processing, splicing, transport or translation of the relevant mRNA. Moreover, certain AS nucleotide sequences can elicit cellular RNase H activity when hybridized with their target mRNA, resulting in mRNA degradation [Calabretta *et al*, 1996]. In that case, RNase H will cleave the RNA component of the duplex and can potentially release the AS to further hybridize with additional molecules of the target RNA. An additional mode of action results from the interaction of AS with genomic DNA to form a triple helix which may be transcriptionally inactive.

Phosphorothioate antisense oligonucleotides do not normally show significant toxicity at concentrations that are effective and exhibit sufficient pharmacodynamic half-lives in animals [Agarwal *et al.*, 1996] and are nuclease resistant. Antisense induced loss-of-function phenotypes related with cellular development were shown for the glial fibrillary acidic protein (GFAP), for the establishment of tectal plate formation in chick [Galileo *et al.*, 1991] and for the N-myc protein, responsible for the maintenance of cellular heterogeneity in neuroectodermal cultures (ephithelial vs. neuroblastic cells, which differ in their colony forming abilities, tumorigenicity and adherence) [Rosolen *et al.*, 1990; Whitesell *et al*, 1991]. Antisense

oligonucleotide inhibition of basic fibroblast growth factor (bFgF), having mitogenic and angiogenic properties, suppressed 80% of growth in glioma cells [Morrison, 1991] in a saturable and specific manner. Being hydrophobic, antisense oligonucleotides interact well with phospholipid membranes [Akhter *et al.*, 1991]. Following their interaction with the cellular plasma membrane, they are actively (or passively) transported into living cells [Loke *et al.*, 1989], and this may occur by a saturable mechanism predicted to involve specific receptors [Yakubov *et al.*, 1989].

Instead of an antisense sequence as discussed herein above, ribozymes may be utilized for suppression of gene function. This is particularly necessary in cases where antisense therapy is limited by stoichiometric considerations [Sarver et al., 1990, Gene Regulation and Aids, pp. 305-325]. Ribozymes can then be used that will target the same sequence. Ribozymes are RNA molecules that possess RNA catalytic ability [see Cech for review] that cleave a specific site in a target RNA. The number of RNA molecules that are cleaved by a ribozyme is greater than the number predicted by stochiochemistry. [Hampel and Tritz, 1989; Uhlenbeck, 1987]. Therefore, the present invention also allows for the use of the ribozyme sequences targeted to the UTR of a housekeeping gene. In an embodiment the R1 UTR or R2 UTR is used. The ribozyme sequence is targeted to segments of the UTR sequence. The ribozymes are made and delivered as discussed herein below. The ribozymes may be used in combination with the UTR oligonucleotide sequences and antisense sequences.

Ribozymes catalyze the phosphodiester bond cleavage of RNA. Several ribozyme structural families have been identified including Group I introns, RNase P, the hepatitis delta virus ribozyme, hammerhead ribozymes and the hairpin ribozyme originally derived from the negative strand of the tobacco ringspot virus satellite RNA (sTRSV) (Sullivan, 1994; U.S. Patent No. 5,225,347, columns 4-5).

The latter two families are derived from viroids and virusoids, in which the ribozyme is believed to separate monomers from oligomers created during rolling circle replication (Symons, 1989 and 1992). Hammerhead and hairpin ribozyme motifs are most commonly adapted for trans-cleavage of mRNAs for gene therapy (Sullivan, 1994). The ribozyme type utilized in the present invention is selected as is known in the art. Hairpin ribozymes are now in clinical trial and are the preferred type. In general the ribozyme is from 30-100 nucleotides in length.

The present invention provides pharmaceutical compositions with the oligonucleotides of the present invention as active ingredients which can also be used in the method of the present invention. Where required the oligonucleotides are nuclease resistant. In general the pharmaceutical composition for inhibiting the tumorigenicity of neoplastic cells in a mammal consists of an effective amount of at least one oligonucleotide from an untranslated region (UTR) of mRNA from a housekeeping gene as described above needed for the practice of the invention or a sequence segment thereof shown to have the same effect or the appropriate antisense sequences and/or ribozymes and a pharmaceutically physiologically acceptable carrier or diluent.

In a preferred embodiment the pharmaceutical composition for inhibiting tumorigenicity of neoplastic cells in a mammal consists of an effective amount of at least one active ingredient selected from oligonucleotides having a sequence corresponding to the entire 3'-UTR of the mRNA for the R1 or R2 component or segments thereof substantially free of the coding sequence of ribonucleotide reductase protein R1 or R2 respectively, or the antisense sequences thereof or ribozymes comprising a sequence complementary to at least a portion of said UTR and a pharmaceutically physiologically acceptable carrier or diluent. Combinations of the active ingredients can be used.

The compositions can be administered orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and infusion techniques as required by the malignant cells being treated. For delivery within the CNS intrathecal delivery can be used with for example an Ommaya reservoir or other methods known in the art. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention. Implants of the compounds are also useful. In general the pharmaceutical compositions are sterile.

In the method of the present invention the neoplastic cells are contacted with a growth inhibiting amount of the bioactive oligonucleotide having the housekeeping gene mRNA UTR sequence of the mammal to be treated or a sequence segment (alternatively designated fragment) thereof shown to have substantially the same effect. These UTR oligonucleotide sequences or sequence segments thereof are substantially free of the coding sequence of the gene and can be formed from ribonucleotides or deoxyribonucleotides. In a preferred embodiment the UTR is a 3'UTR and the mammal is human.

In a preferred embodiment the neoplastic cell is contacted with a growth inhibiting amount of at least one oligonucleotide having a sequence corresponding to the entire 3'-UTR of the mRNA for the R1 or R2 component or segments thereof substantially free of the coding sequence of ribonucleotide reductase protein R1 or R2 respectively. In a further embodiment the antisense sequences thereof or ribozymes comprising a sequence complementary to at least a portion of the 3'-UTR as described herein can be used.

In a further embodiment, an effective amount of ribonucleotide reductase R2 mRNA UTR oligonucleotide sequence substantially free of the coding sequence of the

R2 gene component of ribonucleotide reductase or sequence segments thereof is used in the method of the present invention to reduce or prevent metastasis.

In a further embodiment, a method of inhibiting the tumorigenicity of neoplastic cells resistant to chemotherapeutic compounds such as hydroxyurea in a mammal is provided. The method identifies patients who have tumors that are resistant to hydroxyurea. These patients are then treated with a growth inhibiting amount of at least one active composition from oligonucleotides having a sequence corresponding to the entire 3'-UTR of the mRNA for the R1 or R2 component or segments thereof substantially free of the coding sequence of ribonucleotide reductase protein R1 or R2 respectively or the antisense sequences thereof or ribozymes comprising a sequence complementary to at least a portion of the UTR.

By bioactive (expressible) is meant that the oligonucleotide is biologically active in the cell when delivered directly to the cell and/or is expressed by an appropriate promotor and active when delivered to the cell in a vector as described herein below. Nuclease resistance is provided by any method known in the art that does not substantially interfere with biological activity.

By contacting the cell, it is meant methods of exposing or delivery to a cell of oligonucleotides whether directly or by viral or non-viral vectors and where the oligonucleotide is bioactive upon delivery. The method of delivery will be chosen for the particular cancer being treated based on at least the parameters of cell type affected and location as is known in the medical art.

It is noted that humans are treated generally longer than the Examples exemplified herein, which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses may be single doses or multiple doses as determined by the medical practitioners and treatment courses will be repeated as necessary. Variations in the embodiments used may also be utilized.

The amount must be effective to achieve improvement including but not limited to decreased tumor growth, or tumor size reduction or to improved survival rate or length or other indicators as are selected as appropriate measures by those skilled in the art.

Further, the pharmaceutical compositions utilized in the present invention are administered in combination with other drugs or singly, consistent with good medical practice such as cytotoxic agents, immunotoxins, alkylating agents, anti-metabolites, antitumor antibiotics and other anti-cancer drugs and treatment modalities that are known in the art. The composition is administered and dosed in accordance with good medical practice taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, and other factors known to medical practitioners. The "effective amount" for growth inhibition is thus determined by such considerations as are known in the art. The pharmaceutical composition may contain more than one embodiment of the present invention.

The UTR nucleotide sequences, antisense oligonucleotides and ribozymes of the present invention can be synthesized by any method known in the art for ribonucleic or deoxyribonucleic nucleotides. For example, an Applied Biosystems 380B DNA synthesizer can be used. When oligonucleotide sequence segments of the UTR are used, two or more such oligonucleotide sequence segments can be synthesized and linked together for use in the present invention.

The nucleotide sequences of the present invention can be delivered either directly or with viral or non-viral vectors. When delivered directly the sequences are generally rendered nuclease resistant. Alternatively the sequences can be incorporated into expression cassettes or constructs such that the sequence is expressed in the cell. Generally the construct contains the proper regulatory

sequence or promotor to allow the sequence to be expressed in the targeted cell.

Once the oligonucleotide sequences are ready for delivery they can be introduced into cells as is known in the art. Transfection, electroporation, fusion, liposomes, and viral vectors as well as other means known in the art may be used to deliver the oligonucleotide sequences to the cell. Which method is selected will depend at least on the cells to be treated and the location of the cells and will be known to those skilled in the art. Localization can be achieved by liposomes, having specific markers on the surface for directing the liposome, by having injection directly into the tissue containing the target cells, by having depot associated in spatial proximity with the target cells, specific receptor mediated uptake, viral vectors, or the like.

As discussed herein, the present invention provides vectors comprising an expression control sequence operatively linked to the oligonucleotide sequences of the invention. The present invention further provides host cells, selected from suitable eucaryotic and procaryotic cells, which are transformed with these vectors as necessary. Such transformed cells allow the study of the function and the regulation of malignancy and the treatment therapy of the present invention.

The present invention provides for transgenic parental strains carrying the UTR of the present invention or UTRs constructed to carry UTRs with mutations and where appropriate as well as for knockout models. These models are constructed using standard methods known in the art and as set forth in United States Patents 5,614,396 5,487,992, 5,464,764, 5,387,742, 5,360,735, 5,347,075, 5,298,422, 5,288,846, 5,221,778, 5,175,385, 5,175,384, 5,175,383, 4,873,191, 4,736,866 as well as Burke and Olson (1991), Capecchi (1989), Davies et al. (1992), Dickinson et al. (1993), Duff and Lincoln (1995), Huxley et al. (1991), Jakobovits et al. (1993), Lamb et al. (1993), Pearson and

Choi (1993), Rothstein (1991), Schedl et al. (1993), Strauss et al. (1993). Further, patent applications WO 94/23049, WO 93/14200, WO 94/06908, WO 94/28123 also provide information.

More specifically, any techniques known in the art may be used to introduce the transgene expressibly into animals to produce the parental lines of animals. Such techniques include, but are not limited to, pronuclear microinjection (U.S. patent 4,873,191); retrovirus mediated gene transfer into germ lines [Van der Putten et al., 1985]; gene targeting in embryonic stem cells [Thompson et al., 1989 and U.S. patent 5,614,396]; electroporation of embryos [Lo, 1983]; and sperm-mediated gene transfer [Lavitrano et al., 1989]. For a review of such techniques see Gordon [1989].

Vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the oligonucleotides in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI (1995), Vega et al.,

Gene Targeting, CRC Press, Ann Arbor, MI (1995), *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, Butterworths, Boston MA (1988) and Gilboa et al (1986) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors.

Introduction of the oligonucleotides of the present invention by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

Recombinant methods known in the art can also be used to achieve the sense, antisense or triplex inhibition of a target nucleic acid. For example, vectors containing antisense nucleic acids can be employed to express an antisense message to reduce the expression of the target nucleic acid and therefore its activity.

A specific example of DNA viral vector for introducing and expressing the UTR mRNA R1 and/or R2 sequence is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor which includes most cancers of epithelial origin as well as others. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells including, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy.

Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the anti-viral gancyclovir. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or sequence, cellular transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

Recombinant viral vectors are another example of vectors useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected

cell. The vector to be used in the methods of the invention will depend on the desired cell type to be targeted. For example, if breast cancer is to be treated, then a vector specific for such epithelial cells should be used. Likewise, if cells of the hematopoietic system are to be treated, then a viral vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, should be used.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration may provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration, especially in the case of neuro-

degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

Antibodies may be produced against the oligonucleotides of the present invention. In a preferred embodiment antibodies are made that are directed against oligonucleotides having a sequence corresponding to the entire 3'-UTR of the mRNA for the R1 or R2 component or segments thereof substantially free of the coding sequence of ribonucleotide reductase protein R1 or R2 respectively.

The techniques used to produce antibodies are widely practiced in the art, and most practitioners are familiar with the standard resource materials which describe specific conditions and procedures. However, for convenience, the following paragraphs may serve as a guideline.

Antibodies (immunoglobulins) may be either monoclonal or polyclonal and are raised against the oligonucleotides of the present invention (immunogen). Such immunogens can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992. Antibody fragments may also be prepared from the antibodies and include Fab, $F(ab')_2$, and Fv by methods known to those skilled in the art.

For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the immunogen, generally with an adjuvant and, if necessary, coupled to a carrier;

antibodies to the protein are collected from the sera. Further, the polyclonal antibody can be absorbed such that it is monospecific. That is, the sera can be absorbed against related immunogens so that no cross-reactive antibodies remain in the sera rendering it monospecific.

For producing monoclonal antibodies the technique involves hyperimmunization of an appropriate donor with the immunogen or immunogen fragment, generally a mouse, and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes the oligonucleotides of the invention.

For producing recombinant antibody (see generally Huston et al, 1991; Johnson and Bird, 1991; Mernaugh and Mernaugh, 1995), messenger RNAs from antibody producing B-lymphocytes of animals, or hybridoma are reverse-transcribed to obtain complimentary DNAs (cDNAs). Antibody cDNA, which can be full or partial length, is amplified and cloned into a phage or a plasmid. The cDNA can be a partial length of heavy and light chain cDNA, separated or connected by a linker. The antibody, or antibody fragment, is expressed using a suitable expression system to obtain recombinant antibody. Antibody cDNA can also be obtained by screening pertinent expression libraries.

The antibody or antibody fragment can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow & Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992) The detectable moieties contemplated with the present invention can include, but are not limited to, various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, b-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include radioactive iodine I^{125}, I^{131} or tritium.. Antibodies may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

Antibodies specifically reactive with the oligonucleotides, or analogues thereof, such as enzyme conjugates or labeled derivatives, may be used to detect UTRs in various biological materials, for example they may be used in any known immunoassays. Examples of such assays are radioimmunoassays, enzyme immunoassays (e.g.ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination, and histochemical tests. Thus, the antibodies may be used to detect and quantify UTRs in a sample.

In particular, the antibodies of the invention may be used in immuno-histochemical analyses, for example, at the cellular and subcellular level, to detect UTRs, to localise it to particular cells and tissues and to specific subcellular locations, and to quantitate the level of expression.

Cytochemical techniques known in the art for localizing antigens using light and electron microscopy may be used to detect UTRs. Generally, an antibody of the invention labelled with a detectable substance and UTRs may be localised in tissue based upon the presence of the detectable substance.

Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against the UTRs. By way of example, if the antibody having specificity against the UTR is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labelled with a detectable substance as described herein.

Where a radioactive label is used as a detectable substance, the UTR may be localized by radioautography. The results of radioautography may be quantitated by determining the density of particles in the radioautographs by various optical methods, or by counting the grains.

The presence of an oligonucleotide (or UTR) of the present invention may be indicative of the status of the cell. The absence of the UTR may indicate a potential for tumor growth. Assaying a cell for the presence/absence of the UTRs may be useful in monitoring the progression of a cancer therapy. Such assays may be performed using probes or antibodies that bind the UTRs as described in detail above.

Regulation of mRNA molecules, including message stability and translation efficiency, is mediated by the binding of *trans*-acting factors (generally proteins) with unique *cis*-elements in the untranslated regions of the

message as discussed herein. Methods of identifying these *trans*-acting factors are needed.

Therefore the present invention also includes the use of the oligonucleotides to screen for or to identify substances that bind to the oligonucleotides. Such substances may be antagonists or agonists of the oligonucleotides and can also modulate the tumorigenic properties of a cell either in a positive or negative way. Such substances include nucleic acid sequences and proteins.

Accordingly, the present invention provides a method for identifying a substance that modulates the tumorigenic properties of a cell. A test substance is reacted with an oligonucleotide comprising at least seven consecutive nucleotides or nucleotide analogues of an untranslated region of mRNA of a housekeeping gene, under conditions which permit the formation of complexes between the test substance and oligonucleotide. In an assay, complexes, free substances, non-complexed oligonucleotide are then identified by determining if the test substance binds to the oligonucleotide, and if it modulates tumorigenic properties of a cell.

The substance-oligonucleotide complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques, for example, using UV or chemical crosslinking followed by electrophoresis or chromatography of the crosslinked complexes such as mobility gel shifts. To facilitate the assay of the components, antibody against the oligonucleotide or the substance, or labelled oligonucleotide, or a labelled substance may be utilized. The antibodies, oligonucleotide, or substances may be labelled with a detectable substance as described above.

The oligonucleotide, or the substance used in the method of the invention may be insolubilized. For example,the oligonucleotide or substance may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose,

carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere, etc.

The insolubilized oligonucleotide or substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

The present invention also includes the use of the oligonucleotides to screen for substances that are antagonists or agonists of the binding of the oligonucleotides to substances that bind the oligonucleotides. Accordingly, the present invention provides a method for screening for an agonist or antagonist of the interaction of an oligonucleotide with a substance which binds to the oligonucleotides of the present invention and thereby modulates the tumorigenic properties of a cell. The method includes providing a known concentration of the oligonucleotide and a substance which is capable of binding to the oligonucleotide, and a test substance under conditions which permit the formation of complexes between the substance and oligonucleotide. Undertaking assays for complexes, for free substance, for non-complexed oligonucleotide to determine if the substance is an agonist or antagonist of the interaction of the substance and oligonucleotide, and if it modulates tumorigenic properties of a cell.

It will be understood that the agonists and antagonists that can be assayed using the methods of the invention may act on one or more of the binding sites on the oligonucleotide or substance including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of the interaction of the oligonucleotide with a substance which is capable of binding to the oligonucleotide. Thus, the invention may be used to assay for a substance that competes for the same binding site of the oligonucleotide.

The invention also includes substances identified or isolated using the above described methods. The substances identified by the methods described herein, may be used for modulating the tumorigenic properties of a cell. The substances may be formulated into pharmaceutical compositions as described above for the oligonucleotides.

More specifically, to identify the *trans*-acting proteins, untranslated sequences of the present invention that inhibit tumor cell growth are used to screen for unique mRNA binding proteins in cancer cell extracts. Unique sequences of the present invention are used in the assay. In general, mobility gel shift and UV cross-linking procedures are used [Amara et al, 1993] to identify the presence of the protein and to which sequence it binds. The binding proteins are purified by methods known in the art and may use, for example, affinity purification procedures utilizing an untranslated sequence of the present invention which is attached to sepharose beads. Once the proteins are purified and identified, standard techniques can be used to clone the genes for these proteins. Alternatively, cloning of a cDNA encoding a mRNA binding protein could be accomplished by screening expression libraries with the untranslated sequences using for example the Northwestern procedure [Qian et al, 1993].

The present invention also includes the use of the oligonucleotides corresponding to the entire 3'-UTR of the mRNA for the R1 or R2 component or sequence segments of at least seven consecutive nucleotides thereof substantially free of the coding sequence of ribonucleotide reductase protein R1 or R2 respectively to screen for other mRNAs in

the cell that contain identidal or very similar nucleotide sequences. This porvides information on other targets for oligonucleotide action and the factors that they bind. These targets and factors are then available for use in developing therapeutic interventions. The test is performed, for example, using nucleic acid hybridization or polymerase chain reaction (PCR) techniques known to one skilled in the art.

The oligonucleotides of the invention allow those skilled in the art to construct nucleotide probes for use in the detection of homologous untranslated regions in biological materials. Hence, the probes can be used to screen for other UTRs having tumor modulating, preferably tumor suppressive, activity. Suitable probes include nucleic acid molecules based on nucleic acid sequences from regions of the 3' UTR of ribonucleotide reductase R1 or R2 as shown in SEQ ID Nos:6-49.

A nucleotide probe may be labelled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P, ^{3}H, ^{14}C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and luminescent compounds. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization. Labelled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.).

As shown in the Examples, oligonucleotide sequences of the untranslated regions of ribonucleotide reductase R1 and R2 mRNAs, even though ribonucleotide reductase is a housekeeping activity, can function as regulators of cell growth, both in the development of tumors, tumorigenicity

(3'UTRs of R1 and R2), and in the more complex process of tumor metastasis (3'UTR of R2).

A hypothesis for the observations in the Examples can be made, but it is not to be construed as limiting the present invention to this one mode of action. The mechanism(s) responsible for determining the malignant suppressive effects described herein are presently unknown but they could include RNA-protein, RNA-RNA or perhaps RNA-DNA interactions. However, based upon knowledge of ribonucleotide reductase regulation, several reasonable possibilities can be considered. For example, expression of 3' UTRs of highly regulated messages, like those associated with R1 and R2 mRNAs, could bind proteins or other regulators of cell growth, and therefore reduce the interaction of these factors with their primary targets. Support for this view comes from recent studies that have demonstrated that the R1 and R2 mRNAs bind novel cytoplasmic proteins that control gene expression through changes in message stability. Indeed, the binding of two of these proteins, R1BP and R2BP, to unique *cis*-element sequences of R1 and R2 mRNAs are sensitive to tumor promoter treatment [Amara et al., 1994; Chen et al., 1993; Chen et al., 1994a; Chen et al., 1994b; Amara et al., 1995a], and R1BP binding to its message sequence is mediated through a protein kinase C pathway [Chen et al., 1994a].

It is also possible that expression of non-coding message sequences could modify growth related malignant characteristics by sequestering regulators that are important for the action of growth factors. For example, many malignant cells exhibit profound changes in growth factor regulation of cell proliferation [Wright et al., 1993]. Applicants and others have shown this to be the case for the regulation of tumor cell proliferation in the presence of TGF-β_1 [Nowell, 1986; Wright et al., 1993; Schwarz et al., 1988], where an antisense oligodeoxyribonucleotide directed against TGF-β_1 can

significantly reduce tumorigenic and metastatic potential [Spearman et al., 1994]. Interestingly, Applicants have shown that TGF-β_1 treatment of transformed cells can directly modify ribonucleotide reductase gene expression [Hurta et al., 1991], and this occurs at least in part through changes in message stability regulation [Amara et al., 1995b; Hurta and Wright, 1995], which involves the binding of a protein to the 3' UTR of the R2 message [Amara et al., 1995b].

Applicants propose that expression of the R1 or R2 3' UTRs of ribonucleotide reductase can influence tumorigenic or metastatic properties of cells through interactions with cellular regulators that are important in mechanisms of post-transcriptional control of gene expression. Applicants assume that these regulators normally interact with ribonucleotide reductase message to fine tune the expression of this activity in DNA synthesis and repair, which is involved in cell proliferation [Wright et al., 1990a; Wright, 1989]. It is also reasonable to assume that these regulators could play a positive or negative role in the control of the expression of other genes too, some of which may behave as tumor suppressors or oncogenes.

EXAMPLES

GENERAL METHODS:

GENERAL METHODS IN MOLECULAR BIOLOGY: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992); in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989); and in Perbal, *A Practical Guide to Molecular Cloning*, John Wiley & Sons, New York (1988). Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990).

Vectors can be constructed for the present invention by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. The expression elements can be selected to allow expression only in the cell being targeted. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. One of ordinary skill in the art will know which expression elements are compatible with a particular cell type. The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art as described herein above.

GENERAL METHODS IN IMMUNOLOGY: Standard methods in immunology known in the art and not specifically described were generally followed as in Stites et al.(eds), Basic and Clinical Immunology (8th Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), Selected Methods in Cellular Immunology, W.H. Freeman and Co., New York (1980).

ASSAYS FOR TUMORIGENICITY AND METASTASIS: Malignancy potential was determined as reported previously [Wright, 1989a; Egan et al., 1987a, 1987b; Damen et al., 1989; Taylor et al., 1992; Stokoe et al., 1994]. Six to eight week old C3H/HeN syngeneic mice (Charles River, Quebec) were used to evaluate tumorigenic and metastatic potential of the cells. Cells were prepared from subconfluent, logarithmically growing cultures, collected by gentle treatment with trypsin/EDTA solution and adjusted to appropriate concentration in a balanced salt solution.

For the tumorigenicity (tumor latency) assay, 1×10^5 cells in a 0.1 ml volume were injected subcutaneously into the back of mice and the time required to form a tumor (2 X 2 mm) detectable by palpation was recorded. The growth of tumors was also evaluated by measuring tumor diameters, and estimating tumor base area each day following tumor appearance [Damen et al., 1989]. Tumor size was determined

by multiplying the dimensions of the cross-section of the tumor. Tumors were removed from the mice and tumor weight was recorded 21 days later. In the case of no tumor formation, mice were kept for 2 months after injection and then sacrificed.

For experimental metastasis assays (determination of metastatic potential), 1×10^5 cells in a 0.2 ml volume were injected into the tail veins of 6-8 week old C3H/HeN syngeneic mice and an estimate of the number of lung tumors was made 21 days later. The mice were sacrificed, and the lungs were stained by injecting Bouin's solution {picric acid, formaldehyde, acetic acid (15:5:1)} intratracheally [Egan et al., 1987b; Damen et al., 1989]. Pulmonary tumors were counted with the aid of a dissecting microscope. To confirm that equal numbers of test and control cells were injected, duplicate culture plates containing growth medium were inoculated with 100 cells per plate. After 10 days in culture, plates were stained with methylene blue and colonies were scored.

EXAMPLE 1

Neoplastic transformation is a multi-stage process that usually proceeds through the accumulation of numerous genetic alterations [Nowell, 1986; Wright et al., 1993]. Activation of specific oncogenes and inactivation of tumor suppressor genes play important roles in this mechanism. In previous studies, Applicants have shown that mouse 10T½ fibroblasts transfected with a combination of T24-H-*ras*, human c-*myc* and the proline 193 mutant form of p53 exhibit tumorigenic and metastatic properties in syngeneic mice [Taylor et al., 1992; Huang et al., 1995b]. RMP-6 is one of these highly malignant cell lines that has been characterized in these earlier studies. Ribonucleotide reductase mRNA non-coding regions [Amara et al., 1994; Chen et al., 1993; Chen et al., 1994a; Chen et al., 1994b; Amara et al., 1995a; Amara et al., 1995b], ability to modify

malignancy-related characteristics was tested using the RMP-6 cell line.

In preparation for these experiments, RMP-6 cells were transfected by the calcium phosphate precipitation procedure with expression plasmids containing the R1 3′ UTR (SEQ ID No:1), the R2 3′ UTR (SEQ ID No:2) or with the empty vector as a control, to yield the cell lines RMPM1U, RMPM2U and RMP-VC, respectively. RMP-6 cells were also transfected by electroporation with the same plasmid constructs to produce the cell lines eRMPM1U (R1 3′ UTR), eRMPM2U (R2 3′ UTR) and eRMP-VC (empty vector) (Fig. 1). Cells transfected by either calcium phosphate precipitation or by electroporation express the transfected 3′ UTRs. In addition, an 831 base fragment encoded by chlamydial DNA was expressed in cells transfected with pHNC0.8. The plasmid constructs also contain the coding region for the luciferase enzyme, and as expected all transfected cell lines contained luciferase activity.

MATERIALS AND METHODS

Construction of expression plasmids for the 3′ UTRs of ribonucleotide reductase: A 1854 bp fragment of a recombinant hygromycin gene which contained a mammalian thymidine kinase promoter, the coding region of the hygromycin gene, and a thymidine kinase polyadenylation signal was PCR-amplified from the plasmid pEBVHis (Invitrogen Corp., San Diego, CA), and inserted into the *Bst*1107I site of the mammalian expression plasmid pcDNA3 (Invitrogen Corp.), to give the plasmid pHN. A 1696 base pair (bp) fragment which covered the 1650 bp coding region and 46 bp 3′ UTR of firefly luciferase cDNA was amplified from pMAMneo-luc (Clontech, Palo Alto, CA), and inserted into the *Hind*III and *Kpn*I-restricted pHN. Into the *Kpn*I and *Xho*I sites of the resulting plasmid were inserted the 446 bp fragment of R1 3′ UTR, and the 876 bp fragment of the R2 3′ UTR, amplified from the pCD-R1 and pCD-R2

plasmids [Amara et al., 1994; Chen et al., 1993; Thelander and Berg, 1986], to produce the R1 and R2 3′ UTR expression plasmids, pHNM1U and pHNM2U, respectively.

A control expression plasmid, pHNC0.8 was also constructed by inserting a 831 bp fragment of *Chlamydia trachomatis* genomic DNA into the *BamH*1 and *Xho*I sites of the same vector. The 831 bp chlamydial fragment is a 3′-portion of an open reading frame encoding a thymidylate synthase [Fan et al., 1996C]. In pHNM1U, pHNM2U and pHNC0.8, the synthesis of a recombinant luciferase mRNA, which contains (from 5′ → 3′) the luciferase coding region, 46 bases of luciferase 3′ UTR plus the full length 3′ UTR of R1, R2 or the chlamydial sequence is under the control of a cytomagalovirus promoter.

In addition, the cDNA fragments for R1 or R2 3′UTRs were directionally cloned into *Kpn*I/*Xho*I cut pcDNA3 plasmid to yield expression vectors pD3M1U and pD3M2U, respectively. In these latter two vectors, full length R1 or R2 3′UTR without an upstream luciferase coding fragment is also under the control of a cytomegalovirus promoter. The orientation of the inserts in all the recombinant plasmids was confirmed by sequence analysis using a sequencing kit (Gibco BRL, Burlington, Ontario).

Transfection of plasmid DNA into cells: Expression plasmid DNA was introduced into human Hela cells by calcium phosphate precipitation, and into RMP-6 cells by calcium phosphate precipitation or electroporation [Taylor et al., 1992; Huang et al., 1995b]. For calcium phosphate precipitation, 5×10^5 cells were seeded into 10 cm cell culture dishes containing 10 ml of α-minimal essential medium (Gibco) supplemented with 10% serum (Fetal Clone III, Hyclone, UT). After about 16 hours of culture at 37°C in the presence of 5% CO_2, the medium was changed and cells were cultured a further 3 hours. Twenty ug of DNA was used for transfection of each dish of cells; the DNA-phosphate precipitates were prepared as previously described [Taylor et al., 1992; Huang and Wright, 1994]. After 16 hours of

cell culture, the precipitates were removed and cells were washed twice with phosphate buffered saline, pH 7.2 and fresh medium was added for overnight culture. RMP-6 cells were then cultured in medium containing 400 μg/ml hygromycin (Boehringer-Mannheim, Mannheim, Germany), and Hela cells were cultured in medium containing 800 μg/ml geneticin (Gibco). Selected stable transfectant colonies (more than 500 in total) were identified, removed with trypsin solution, pooled and cultured in the selective medium for another 10 days to ensure that they were drug resistant [Huang and Wright, 1994].

For electroporation, the expression plasmids were linearized with *Ssp*1, extracted with phenol:chloroform, precipitated with ethanol and redissolved in serum-free medium containing 10 mM Hepes (pH 7.2). Logarithmically growing cells were removed with trypsin solution, and washed twice with 10 mM Hepes (pH 7.2). An electroporation mixture was prepared in an electroporation cuvette and contained, in a total volume of 400 μl, 7 x 10^6 cells and 20 μg of *Ssp*1-digested plasmid DNA. The electroporation was achieved by using a Gene Pulser (Bio-Rad, Mississauga, ON) with settings at 960 μF and 250 V. After 5 minutes incubation at room temperature, the cells were transferred into a 10 cm culture dish containing 15 ml of growth medium. After overnight culture, hygromycin was used to select for stable transfectants [Huang and Wright, 1994].

Reverse transcriptase PCR (RT-PCR): Total cellular RNA was extracted from approximately 70% confluent cultures by using a Micro RNA Isolation Kit as instructed by the manufacturer (Strategene, La Jolla, CA). An Sp6 primer (5'GGATTTAGGTGACACTATAG3', SEQ1 ID No:3) located 20 bp downstream of the *Xho*I-cutting site of the vector (where the R1 or R2 3' UTRs were cloned) was used for reverse transcription from the recombinant mRNAs containing the UTRs. A second primer (5'TGAGAAAAGCGGGGCCTG3', SEQ ID No:4), which is the first 18 bp of the R1 3' UTR, and a third primer (5'TAAGTAACTGATCGTGTGCTC3', SEQ ID No:5),

which represents the first 21 bp of the R2 3' UTR was used in combination with the Sp6 primer to amplify the recombinant cDNAs. A chlamydial DNA primer (5'TTAAGACTTTTTACGCGATTC3', SEQ ID No:50) was used together with the Sp6 primer to detect expression of the bacterial fragment in pHNC0.8 transfected cells.

An EZ rTth RNA PCR Kit (Perkin Elmer, Branchburg, NJ) was used for the RT-PCR. Briefly, an RT-PCR reaction contained, in a total volume of 50 μl, 1× EZ buffer (Perkin Elmer), 300 μM of each deoxyribonucleoside triphosphate, 2.5 mM $Mn(OAc)_2$, 100 ng of RNA template, 0.45 μM each of two primers and 5.0 units of rTth Polymerase. To ensure that the final amplification product was initially amplified from RNA instead of a possible DNA contaminant in the RNA samples, a parallel reaction was carried out, in which 1 μg of DNase-free RNase A was added into, and incubated with, the reaction mixture for 5 minutes before the addition of the polymerase.

The synthesis of cDNA from template mRNA and later amplification of the cDNA was achieved by incubation at 60°C for 60 minutes, then 94°C for 2 minutes followed by 40 temperature cycles of 20 seconds of denaturing at 94° C, 90 seconds of annealing and extension at 60°C, and a final 7 minutes incubation at 60°C. At the end of the reaction, 10 μl of sample was analyzed by electrophoresis on 1% agarose gel.

Tumorigenicity and metastasis analysis: Malignant potential was determined as described herein above.

RESULTS

To evaluate the possibility that expression of the ribonucleotide reductase 3' UTRs affect tumorigenicity, syngeneic mice were injected subcutaneously with the transfected cell lines and tumor weight and growth was determined. Metastatic potential in syngeneic mice was estimated by a tail vein experimental lung metastasis assay. Cells expressing R1 and R2 3' UTRs produced subcutaneous tumors that were significantly reduced in

weight when compared to results obtained with control cells that were transfected with the empty vector (Table 1). Similar results were obtained with cells that were transfected with the calcium phosphate precipitation procedure and with cells that were transfected by electroporation (Table 1). In keeping with these observations, Fig. 2 shows that the growth of tumor cells transfected with the 3' UTRs from R1 or R2 mRNAs was significantly slower than the growth of cells transfected with the vector alone.

To further examine specificity in the reduced tumorigenicity observed with cells expressing R1 or R2 3'UTRs, the tumorigenicity of cells transfected with pHNC0.8 which express 831 bases of chlamydial sequence were compared with cells transfected with the empty vector. There was no significant difference between the two cell populations as determined by estimating tumor weight (Table 1) or tumor latency (data not shown). Cancer mortality is primarily caused by the ability of tumor cells to metastasize [Nowell, 1986]. Interestingly, cells expressing the R2 3' UTR (SEQ ID No:2) exhibited a significantly reduced ability to disseminate to the lungs of syngeneic animals as compared to control cells transfected with the vector alone (Table 1). Expression of the R1 3' UTR (SEQ ID No:1) did not significantly alter metastatic potential when compared to the control population (Table 1), indicating that expression of the R1 3' UTR suppresses tumorigenic but not metastatic potential.

The R2 3' UTR (SEQ ID No:2) exhibited both tumorigenic and metastatic suppressive effects, and as was observed in the tumorigenic studies, the metastatic properties of the transfectants were essentially independent of the method that was used to perform the transfections.

To determine whether or not the mouse R1 and R2 3'UTRs can suppress human tumor cell potential, Hela cells were transfected by calcium phosphate precipitation with the expression vectors containing either the R1 3'UTR (Hela M1U

cells) or the R2 3'UTR (Hela M2U cells). As was observed with mouse tumor cells, growth of Hela M1U and Hela M2U cells was significantly reduced when compared to control Hela cells containing the expression vector without R1 or R2 3'UTR sequences (Table 2).

EXAMPLE 2

FURTHER RESULTS USING THE R1 AND R2 UNTRANSLATED REGIONS

Using the methods herein above, oligonucleotides of R1 and R2 3'-UTR mRNA segments as set forth in Tables 4 and 5 were screened for tumor cell cytotoxicity in relative colony forming efficiency experiments [Huang and Wright, 1994]. Hela S3 and Hela 1mM tumor cells were used as well as a variety of human cancer cell lines as noted in Tables 6 and 7. The cells were cultured for 24 hours at 37°C in growth medium with 10% fetal bovine serum. The cells were washed in 5ml phosphate buffered saline, pH 7.2, once prior to lipofectin +/- oligonucleotide treatment.

The oligonucleotides being tested were added to cell cultures in the presence of 2.5 μg of DOTMA/DOPE (Lipofectin; Life Technologies, Inc.) for four hours. The oligonucleotide was tested at 0.2 μM unless otherwise indicated. Controls were the cultures treated with lipofectin but without the oligonucleotide. After 4 hours the medium containing the oligonucleotide was removed and washed with 5 ml of growth medium. The cells were then cultured in growth medium containing 10% fetal bovine serum for seven to ten days. In some experiments cell aliquotes were removed from the culture and viability was determined using trypan blue exclusion test [Phillips, 1973]. Results were analyzed as percent of surviving cells compared to control cells.

A short oligodeoxyribonucleotide phosphorothioate sequence, Sen-II-2229B-20 (SEQ ID No:7; Table 5) was used to inhibit the proliferation of human tumor cells (Hela) in relative colony forming efficiency experiments. Hela S3

cells (American Type Culture Collection, Rockville, Maryland, U.S.; ATCC) and a Hela cell line (Hela 1 mM) previously selected for resistance to the antitumor agent hydroxyurea [Wright et al., 1987] were used in these experiments (Table 6). Clearly, Sen-II-2229B-20 is a very effective inhibitor of human tumor cell colony forming ability. It is also effective in inhibiting the proliferation of human tumor cells that exhibit resistance to hydroxyurea, a chemotherapeutic compound of clinical significance.

Sen-II-2229B-20 (SEQ ID No:7) and Sen-II-2229A-20 (SEQ ID No:6) are alternative sequences, with 2229A chosen from the version of R2 in GENBANK (submitted by Pavloff) and 2229B chosen from the version published by Pavloff et al. The two sequences provided similar results.

Sen-II-2229B-20 (SEQ ID No:7) and six other 20-mer oligodeoxyribonucleotide sequences (SEQ ID Nos:6,8-12) corresponding to sequence segments (fragments) of the 3'-UTR of R2 and one corresponding to the 3'-UTR of R1 (SEQ ID No:45; Table 4), were tested in relative colony forming efficiency experiments to determine inhibitory effects using a variety of human cancer cells. The results showing estimated percent inhibition of relative colony forming abilities of these various oligonucleotides are provided in Table 7. Clearly, all the compounds were effective antitumor agents against human cancer cells derived from the bladder, colon, lung, breast and pancreas.

Furthermore, analysis of Hela S3 and WI38 (normal strain) cell viability by the trypan blue exclusion test three days after oligonucleotide exposure indicated that Hela S3 tumor cells were approximately three times more sensitive to the cytotoxic effects of Sen-II-2229B-20 oligonucleotide than normal non-tumorigenic WI38 cells averaged over 4-8 determinations.

Throughout this application, various publications, including United States patents and published patent applications are referenced by author and year or number.

Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

TABLE 1.
TUMORIGENICITY AND METASTATIC POTENTIAL OF RMP-6 TRANSFECTED CELL LINES.

Cell Line	Transfected R1 or R2 mRNA 3' UTR	Tumorigenicity: Subcutaneous Tumor Weight (g) (mean±SE)[1]	Experimental Lung Metastases: Number (mean±SE)[2]
RMP-VC	-	1.05 ± 0.22 (n = 10)[3]	17.3 ± 6.8 (n = 10)[3]
RMPM1U	R1	0.58 ± 0.21 (n = 10)	20.0 ± 9.7 (n = 10)
RMPM2U	R2	0.13 ± 0.14 (n = 10)	6.4 ± 5.4 (n = 10)
eRMP-VC	-	1.21 ± 0.14 (n = 5)	20.5 ± 6.7 (n = 5)
eRMPM1U	R1	0.3 ± 0.31 (n = 5)	16.1 ± 6.1 (n = 5)
eRMPM2U	R2	0.18 ± 0.11 (n = 5)	9.2 ± 2.3 (n = 5)

[1] Using Student's t-test the differences in the tumorigenicity results obtained with RMPM1U and RMPM2U cells were found to be statistically significant, when compared to the results obtained with RMP-VC cells, with p values of < 0.02 and < 0.001, respectively. Similarly, the tumorigenicity results obtained with eRMPM1U and eRMPM2U cells were significantly different from the results obtained with eRMP-VC cells, with p values of < 0.01 in both cases.

[2] Using Student's t-test the numbers for experimental metastases obtained with RMPM1U and eRMPM1U cells were not found to be statistically different from the results obtained with the RMP-VC or the eRMP-VC control cell populations, respectively. However, the numbers for experimental metastases observed with RMPM2U and eRMPM2U cells were significantly different when compared to the observations obtained with RMP-VC or eRMP-VC cells, with p values of < 0.02 in both cases.

[3] The number of animals/experiment is shown in ( ).

[4] As an added control for specificity the tumorigenicity of RMP-VC cells was compared to cells transfected with pHNC0.8 to produce the RMPC0.8 cell line, which expresses a 831 base chlamydial sequence (see Fig. 1). No significant difference was observed; tumor weights of 1.19 ± 0.26 (n = 8) and 1.24 ± 0.33 (n = 7) for RMP-VC and RMPC0.8 cells, respectively (p value > 0.5).

TABLE 2.

TUMORIGENIC POTENTIAL OF HUMAN HELA TRANSFECTED CELL LINES

Cell Line	Transfected R1 or R2 mRNA 3'UTR	Tumorigenicity Subcutaneous Tumor Weight (g), mean ± SE[1]
Hela-VC	-	0.177 ± 0.026
Hela M1U	R1	0.055 ± 0.016
Hela M2U	R2	0.072 ± 0.033

[1] Using Student's t-test the differences in the tumorigenicity results obtained with Hela M1U and Hela M2U cells were found to be statistically significant, when compared to the results obtained with Hela-VC cells, with p values of < 0.01 and < 0.05, respectively. The number of animals/experiment was 5.

TABLE 3

PARTIAL LISTING OF HOUSEKEEPING GENES

Genes indicated by an * have been observed to be altered in cancer cells.

a) Nucleic acid metabolism

Ribonucleotide reductase*
Carbamoyl-phosphate synthetase II*
Aspartate carbamoyltransferase*
Dihydroorotase*
Dihyrofolate reductase*
CTP synthetase*
Thymidylate synthetase*
Deoxycytidylate deaminase*
Uridine-cytidine kinase*
Deoxycytidine kinase*
Thymidine kinase*
DNA polymerase*
DNA nucleotidyltransferases*
RNA polymerases*
tRNA methylase*
Dihydrouracil dehydrogenase*
Formylglycinamidine ribonucleotide synthetase*
IMP dehydrogenase*
GMP synthetase*
AMP deaminase*
Adenylate kinase*

b) Carbohydrate metabolism

Hexokinase*
Phosphofructokinase*
Pyruvate kinase*
Glucose-6-phosphatase*
Fructose-1,6-diphosphatase*
Phosphoenolpyruvate carboxykinase*
Pyruvate carboxylase*
Fructokinase*
Glucokinase*
Thiokinase*
Aldolase*
glyceraldehyde-phosphate dehydrogenase*

c) Protein and amino acid metabolism

Glutamate dehydrogenase*
Glutamate-oxaloacetate transaminase*
Tryptophan pyrrolase*
Glutaminase*
5-Hydroxytryptophan decarboxylase

d) Lipid metabolism

Acetyl-CoA carboxylase
α-Glycerophosphate dehydrogenase*
Hydroxymethylglutaryl-CoA synthase*

e) Other metabolic activities

Ornithine decarboxylase*
Ornithine carbamoyltransferase*
cAMP phosphodiesterase*
Adenylate cyclase*
S-Adenosylmethionine synthetase*
Citrate synthase
Aconitase
Isocitrate dehyrogenase
Succinyl-CoA snythase
Succinate Dehydrogenase
Fumerase
NADH dehydrogenase

TABLE 4

R1 UTR Sequence Segments Designed To Inhibit Tumor Cell Growth

SEQ ID No:							
SEQ ID No:44	Sen-I-2627-20	AGTGGGTTTGCTTGAGGTGG	53.9	-39.1	✓	✓	✓
SEQ ID No:45	Sen-I-2650-20	GGCTTTGCTGGACCCTGTTG	56.8	-40.9	✓	✓	✓
SEQ ID No:46	Sen-I-2767-20	AAAAAAAGAAAAAAAAAACG	44.0	-36.6	✓	✓	✓
SEQ ID No:47	Sen-I-2804-20	AGTAGAAGTTTTAGGAATGC	40.1	-33.1	✓	✓	✓
SEQ ID No:48	Sen-I-2863-20	GTTTCATCACCCATTTAGCA	47.5	-35.8	✓	✓	✓
SEQ ID No:49	Sen-I-2923-20	TTTACTGCTTTGACTGGTGG	47.8	-35.7	✓	✓	✓

TABLE 5

	R2 UTR Sequence Segments Designed To Inhibit Tumor Cell Growth						
SEQ ID No:	**Name***	**Sequence 5' - 3'**	**Tm[1] °C**	**dG[2] KCal/ mol**	**D[3]**	**H[4]**	**A[5]**
SEQ ID No:6	Sen-II-2229A-20	GAGTTTTCATATGTGGGAGC	46.1	-35.2	X	✓	✓
SEQ ID No:7	Sen-II-2229B-20	GAGTTTTCTCATATGTGGGA	43.7	-33.7	X	X	✓
SEQ ID No:8	Sen-II-1364-20	AATGAACTGAAGATGTGCCC	48.9	-36.2	✓	✓	✓
SEQ ID No:9	Sen-II-2083-20	AGGAATCTCTCAGGGCAAGG	52.3	-39.0	✓	✓	✓
SEQ ID No:10	Sen-II-1791-20	GCTTGATTTATTTGGTTTCT	43.4	-34.9	✓	✓	X
SEQ ID No:11	Sen-II-1992-20	GCCAGATAGAAGACAGGTTG	46.2	-35.0	✓	✓	✓
SEQ ID No:12	Sen-II-2019-20	ATCCTGTGGCTTGTGTAGTG	47.2	-34.7	✓	✓	✓
SEQ ID No:13	Sen-II-1396-20	TTTTTTTTTTCCATCTCATA	42.3	-34.3	✓	✓	✓
SEQ ID No:14	Sen-II-1561-20	CTGGCTGGCTGTGACTTACC	52.2	-37.8	✓	✓	✓
SEQ ID No:15	Sen-II-1772-20	ACTCACGGCGGCGATAATAG	54.4	-40.8	✓	✓	✓

SEQ ID No:16	Sen-II-1818-20	ATACATTCTCCTGACCACTA	40.5	-32.1	✓	✓	✓
SEQ ID No:17	Sen-II-2007-20	GGTTGTGTTTTTATCCTGTG	44.5	-34.1	✓	✓	✓
SEQ ID No:18	Sen-II-2013-20	GTTTTTATCCTGTGGCTTGT	46.1	-35.6	✓	✓	✓
SEQ ID No:19	Sen-II-2024-20	GTGGCTTGTGTAGTGTCCTG	47.6	-34.5	✓	✓	✓
SEQ ID No:20	Sen-II-2060-20	CTGAGTAGAGTGTTGTGGGA	44.3	-33.0	✓	✓	✓
SEQ ID No:21	Sen-II-2069-20	GTGTTGTGGGATAAAGGAAT	45.5	-35.2	✓	✓	✓
SEQ ID No:22	Sen-II-2180-20	TCTCACTGTATTTTCCTCAA	41.8	-32.6	✓	✓	✓
SEQ ID No:23	Sen-II-2373-20	GGTGTAAGTAGGTTGTGTGA	41.7	-32.0	✓	✓	✓
SEQ ID No:24	Sen-II-2079-20	ATAAAGGAATCTCTCAGGGC	46.9	-36.7	✓	✓	X
SEQ ID No:25	Sen-II-1771-20	TACTCACGGCGGCGATAATA	53.4	-40.1	✓	✓	X
SEQ ID No:26	Sen-II-1581-20	ATAGCAGTGACAATGGCAGT	47.0	-34.9	✓	✓	✓
SEQ ID No:27	Sen-II-1575-20	CTTACCATAGCAGTGACAAT	41.6	-32.7	✓	✓	X
SEQ ID No:28	Sen-II-1499-29	GCTACCTCACAACCAGTCCT	48.1	-35.7	✓	✓	X
SEQ ID No:29	Sen-II-1386-20	ACTTGGCTGATTTTTTTTT	45.7	-36.6	✓	✓	X

SEQ ID No:30	Sen-II-1560-21	CCTGGCTGGCTGTGACTTACC	56.0	-40.9	✓	✓	✓
SEQ ID No:31	Sen-II-1818-21	ATACATTCTCCTGACCACTAA	43.0	-34.0	✓	✓	X
SEQ ID No:32	Sen-II-1536-22	GTAGTATCACCTTTTGCCAGAA	48.3	-37.8	✓	✓	X
SEQ ID No:33	Sen-II-1989-23	GGTGCCAGATAGAAGACAGGTTG	54.4	-41.3	✓	✓	✓
SEQ ID No:34	Sen-II-1361-24	CTAAATGAACTGAAGATGTGCCCT	53.5	-42.2	✓	✓	X
SEQ ID No:35	Sen-II-1791-24	GCTTGATTTATTTGGTTTCTACAC	49.2	-40.3	✓	✓	X
SEQ ID No:36	Sen-II-2079-25	ATAAAGGAATCTCTCAGGGCAAGGA	57.6	-46.8	✓	✓	✓
SEQ ID No:37	Sen-II-2432-25	ATTTTTTATTATCTATGTTCTTCTA	41.8	-37.6	✓	✓	X
SEQ ID No:38	Sen-II-1381-27	CCCTTACTTGGCTGATTTTTTTTTTCC	59.6	-51.9	✓	✓	X
SEQ ID No:39	Sen-II-2060-29	CTGAGTAGAGTGTTGTGGGATAAAGGAAT	57.6	-48.9	✓	✓	✓
SEQ ID No:40	Sen-II-2162-38	AAGCCGTTTCATTTTATTTCTCACTGTATTTTCCTCAA	66.8	-67.9	✓	✓	✓
SEQ ID No:41	Sen-II-2362-34	TAGTTTTGTTTGGTGTAAGTAGGTTGTGTGAGTT	60.8	-55.2	✓	✓	✓
SEQ ID No:42	Sen-II-2463-37	ACCTGTAGTTCATAAAAAAAAAAAAAAAAAAAAAAA	60.7	-64.5	✓	✓	X
SEQ ID No:43	Sen-II-2007-39	GGTTGTGTTTTTATCCTGTGGCTTGTGTAGTGTCCTGGG	72.9	-71.6	✓	✓	X

FOOTNOTES FOR TABLES 4 AND 5

* Name includes the following:

Sen = sense
I = R1
or
II = R2

The first number is the first nucleotide position in the R2 mRNA sequence.

The second number is the length of the sequence segment.

[1] Tm °C = Melting temperature of oligonucleotide duplex formed

[2] dG = Free energy values for oligonucleotide-complement dimer formation

[3] D = Estimate of potential dimer former (✓ = no potential; X = some potential)

[4] H = Estimate for potential self-complementary interations (✓ = no potential; x = some potential)

[5] A = Estimate for potential to bind to sequences in the R1 or R2 messages (✓ = no potential; X = some potential)

The estimates were determined by using the computer modeling program OLIGO Primer Analysis Software, Version 3.4 (distributed by National Biosciences). The program allows the determination of a qualitative estimation of these three parameters and ranks them as "no potential" or "some potential" or "essentially complete potential". Segments were generally selected that had estimates of no potential in all three parameters. However, several segments as shown in Table 5 had parameters that were in the "some potential" category and were still effective having a reduced (some) potential. A balance of the parameters is used in the selection.

TABLE 6

DOSE DEPENDENT REDUCED COLONY FORMING EFFICIENCY FOLLOWING TREATMENT WITH R2 UTR Sen-II-2229B-20

CELL LINE: Hela S3

	Concentration	% Inhib.
Exp. 1	0	-
	0.05 μM	50%
	0.10 μM	55%
	0.20 μM	88%
Exp. 2	0	-
	0.02 μM	-
	0.05 μM	20%
	0.10 μM	48%
	0.2 μM	80%

CELL LINE: Hela 1 mM

Concentration	% Inhib.
0	-
0.05 μM	-
0.10 μM	50%
0.20 μM	85%

Table 7: Reduced Relative Colony Forming Efficiency Following Treatment with 0.2 μM of Various Oligodeoxyribonucleotide Phosphorothioates Corresponding to the Untranslated Regions of R1 or R2 mRNAs[a]

Cell Lines/ Oligos	Sen-II-1364-20	Sen-II-1791-20	Sen-II-1992-20	Sen-II-2019-20	Sen-II-2083-20	Sen-II-2229A-20	Sen-II-2229B-20	Sen-I-2650-20
T24 Human Bladder Carcinoma	60%	50%	60%	60%	60%	50%	50%	60%
HCT116 Human Colon Carcinoma	85%	70%	70%	80%	85%	55%	80%	65%
A549 Human Lung Carcinoma	90%	80%	80%	80%	90%	65%	85%	75%
MDA-MB-231 Human Breast Adenocarcinoma	80%	80%	80%	80%	80%	55%	70%	70%
MIAPaCa-2 Human Pancreatic Carcinoma	70%	60%	65%	65%	70%	70%	60%	80%
CIPAC-1 Human Pancreatic Adenocarcinoma	55%	55%	55%	65%	55%	ND	55%	ND

REFERENCES

Agarwal et al., 1991. Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice. Proc. Natl. Acad. Sci. USA 88:7595-7599.

Agrawal, 1996. Antisense oligonucleotides: towards clinical trials, TIBTECH, 14:376.

Akhter et al, 1991. Interactions of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes). Nuc. Res. 19:5551-5559.

Amara et al., 1994. Phorbol ester modulation of a novel cytoplasmic protein binding activity at the 3'-untranslated region of mammalian ribonucleotide reductase R2 mRNA and role in message stability. J. Biol. Chem. 269:6709-7071.

Amara et al., 1995A. Altered regulation of message stability and tumor promoter-responsive *cis-trans* interactions of ribonucleotide reductase R1 and R2 messenger RNAs in hydroxyurea-resistant cells. Cancer Res. 55:4503-4506.

Amara et al., 1995B. Defining a novel *cis* element in the 3'-untranslated region of mammalian ribonucleotide reductase component R2 mRNA: Role in transforming growth factor-β_1 induced mRNA stabilization. Nucleic Acids Res. 23:1461-1467.

Amara et al. 1996. Defining a novel *cis*-element in the 3'-untranslated region of mammalian ribonucleotide reductase component R2 mRNA: *cis-trans* interactions and message stability. J. Biol. Chem. 271:20126-20131.

Ashihara and Baserga, 1979. Cell Synchronization. Methods Enzymol. 58:248-262.

Blaesse, 1997. Gene Therapy for Cancer. Scientific American 276(6):111-115.

Björklund et al., 1993. Structure and promoter characterization of the gene encoding the large subunit (R1 Protein) of mouse ribonucleotide reductase. Proc. Natl. Acad. Sci. USA 90:11322-11326.

Burke and Olson, "Preparation of Clone Libraries in Yeast Artificial-Chromosome Vectors" in Methods in Enzymology, Vol. 194, "Guide to Yeast Genetics and Molecular Biology", eds. C. Guthrie and G. Fink, Academic Press, Inc., Chap. 17, pp. 251-270 (1991).

Calabretta, et al, 1996. Antisense strategies in the treatment of leukemias. Semin. Oncol. 23:78.

Caceres and Kosik, 1990. Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. Nature, 343:461.

Capecchi, "Altering the genome by homologous recombination" Science 244:1288-1292 (1989).

Caras, 1985. Cloned Mouse Ribonucleotide Reductase Subunit M1 cDNA Reveals Amino Acid Sequence Homology with *Escherichia coli* and Herpesvirus Ribonucleotide Reductases. Biol Chem. 260:7015-7022.

Chan et al., 1993. Phosphorylation of ribonucleotide reductase R2 protein: *in vivo* and *in vitro* evidence of a role for $p\delta 4^{cdc2}$ and CDK2 protein kinases. Biochemistry 32:12835-12840.

Chen et al., 1993. Mammalian ribonucleotide reductase R1 mRNA stability under normal and phorbol ester stimulating conditions: involvement of a *cis-trans* interaction at the 3'-untranslated region. EMBO J., 12:3977-3986.

Chen et al., 1994A. Regulation of mammalian ribonucleotide reductase R1 mRNA stability is mediated by a ribonucleotide reductase R1 mRNA 3'-untranslated region *cis-trans* interaction through a protein kinase C-controlled pathway. Biochem. J. 302:125-132.

Chen et al., 1994B. Defining a novel ribonucleotide reductase R1 mRNA *cis* element that binds to an unique cytoplasmic *trans*-acting protein. Nucleic Acids Res., 22:4796-4797.

Choy et al., 1988. Molecular mechanisms of drug resistance involving ribonucleotide reductase: hydroxyurea resistance in a series of clonally related mouse cell lines selected in the presence of increasing drug concentrations. Cancer Res. 48:2029-2035.

Cifone and Fidler, 1981. Increased metastatic potential is associated with increasing genetic instability of clones isolated from murine neoplasms. Proc. Natl. Acad. Sci. USA 78:6949-6952.

Crooke, 1995. Progress in antisense therapeutics, Hematol. Pathol. 2:59.

Damen et al., 1989. Generation of metastatic variants in populations of mutator and amplificator mutants. J. Natl. Cancer Inst. 81:628-631.

Davis et al., 1994. Purification, Characterization, and Localization of Subunit Interaction Area of Recombinant Mouse Ribonucleotide Reductase R1 Subunit. Biol. Chem. 269:23171-23176.

Davies et al., "Targeted alterations in yeast artificial chromosomes for inter-species gene transfer", Nucleic Acids Research, Vol. 20, No. 11, pp. 2693-2698 (1992).

Dickinson et al., "High frequency gene targeting using insertional vectors", Human Molecular Genetics, Vol. 2, No. 8, pp. 1299-1302 (1993).

Duff and Lincoln, "Insertion of a pathogenic mutation into a yeast artificial chromosome containing the human APP gene and expression in ES cells", Research Advances in Alzheimer's Disease and Related Disorders, 1995.

Eckstein 1985. Nucleoside Phosphorothioates. Ann. Rev. Biochem. 54:367-402.

Egan, et al., 1987A. Expression of H-*ras* Correlates with Metastatic Potential: Evidence for Direct Regulation of the Metastatic Phenotype in 10T1/2 and NIH 3T3 Cells. Mol. Cell. Biol. 7:830-837.

Egan et al., 1987B. Transformation by oncogenes encoding protein kinases induces the metastatic phenotype. Science 238:202-205.

Eriksson et al., 1984. Cell cycle-dependent regulation of mammalian ribonucleotide reductase. The S phase-correlated increase in subunit M2 is regulated by *de novo* protein synthesis. J. Biol. Chem. 259:11695-11700.

Fan et al., 1996A. Ribonucleotide reductase R2 component is a novel malignancy determinant that cooperates with activated oncogenes to determine transformation and malignant potential. Proc. Natl. Acad. Sci. USA 93:14036-14040.

Fan et al., 1996B. A link between ferritin gene expression and ribonucleotide reductase R2 protein, as demonstrated by retroviral vector mediated stable expression of R2 cDNA. FEBS Lett. 382:145-148.

Fan et al., 1996C. Cloning of a gene from *Chlamydia trachomatis* that complements thymidylate synthase-deficient *Escherichia coli*. *In:* Abstracts of the 94th General Meeting of the American Society for Microbiology, p. 134.

Farrell and Lukens, 1995. Naturally occurring antisense transcripts are present in chick embryo chondrocytes simultaneously with the down-regulation of the α1 (I) collagen gene. J. Biol. Chem. 270:3400-3408.

Filatov et al., 1996. Induction of the mouse ribonucleotide reductase R1 and R2 genes in response to DNA damage by UV light. J. Biol. Chem. 271:23698-23704.

Ford et al., 1997. The poly (A) tail inhibits the assembly of a 3'-to 5' exonuclease in an *in vitro* RNA stability system. Mol. Cell. Biol. 17:398-406.

Galileo et al., 1991. J. Cell. Biol., 112:1285.
Gewirtz, 1993. Oligodeoxynucleotide-based therapeutics for human leukemias, Stem Cells Dayt. 11:96.

Gilboa et al., 1986. Transfer and expression of cloned genes using retroviral vectors. BioTechniques 4(6):504-512.

Gordon, 1989. Transgenic Animals. Intl. Rev. Cytol. 115:171-229.

Hake and Richer, 1997. Translation regulation of maternal mRNA. Biochim. Biophys. Acta 1332:M31-M38.

Hampel and Tritz, 1989. RNA Catalytic Properties of the Minimum (-) sTRSV Sequence. Biochemistry 28:4929-4933

Hanania, et al 1995. Recent advances in the application of gene therapy to human disease. Am. J. Med. 99:537.

Huston et al, 1991 "Protein engineering of single-chain Fv analogs and fusion proteins" in Methods in Enzymology (JJ Langone, ed.; Academic Press, New York, NY) 203:46-88.

Huang and Wright, 1994. Fibroblast growth factor mediated alterations in drug resistance, and evidence of gene amplification. Oncogene 9:491-499.

Huang et al., 1995A. Drug resistance and gene amplification potential regulated by transforming growth factor β_1 gene expression. Cancer Res. 55:1758-1762.

Huang et al., 1995B. Multiple effects on drug sensitivity, genome stability and malignant potential by combinations of H-*as*, c-*myc* and mutant p53 gene overexpression. Int. J. Oncol. 7:57-63.

Hurta, et al., 1991. Early induction of ribonucleotide reductase gene expression by transforming growth factor β_1 in malignant H-*ras* transformed cell lines. J. Biol. Chem. 266:24097-24100.

Hurta and Wright, 1992. Alterations in the activity and regulation of mammalian ribonucleotide reductase by chlorambucil, a DNA damaging agent. J. Biol. Chem. 267:7066-7071.

Hurta and Wright, 1995. Malignant transformation by H-*ras* results in aberrant regulation of ribonucleotide reductase gene expression by transforming growth factor-β_1. J. Cell. Biochem. 57:543-556.

Huxley et al., "The human HPRT gene on a yeast artificial chromosome is functional when transferred to mouse cells by cell fusion", Genomics, 9:742-750 (1991).

Iyer et al. 1990. J. Org. Chem. 55:4693-4699.

Jakobovits et al., "Germ-line transmission and expression of a human-derived yeast artificial chromosome", Nature, Vol. 362, pp. 255-261 (1993).

Jensen et al., 1994. Identification of genes expressed in premalignant breast disease by microscopy-directed cloning. Proc. Natl. Acad. Sci, USA. 91:9257-9261.

Johnson and Bird, 1991 "Construction of single-chain Fvb derivatives of monoclonal antibodies and their production in *Escherichia coli* in Methods in Enzymology (JJ Langone, ed.; Academic Press, New York, NY) 203:88-99.

Kimelman and Kirschner, 1989. An antisense mRNA directs the covalent modification of the transcript encoding fibroblast growth factor in *Xenopus* oocytes. Cell 59:687-696.

Klausner and Hartford, 1989. *Cis-trans* models for post-transcriptional gene regulation. Science 246:870-872.

Lamb et al., "Introduction and expression of the 400 kilobase *precursor amyloid protein* gene in transgenic mice", Nature Genetics, Vol. 5, pp. 22-29 (1993).

Lavitrano et al, 1989. Cell 57:717-723

Lee et al., 1993. The *C. Elegans* heterochronic gene *lin*-4 encodes small RNAs with antisense complementarity to *lin*-14. Cell 75:843-854.

Lefebvre-d'Hellencourt et al, 1995. Immunomodulation by cytokine antisense oligonucleotides. Eur. Cytokine Netw. 6:7.

Lev-Lehman et al., 1997. Antisense Oligomers *in vitro* and *in vivo*. In Antisense Therapeutics, A. Cohen and S. Smicek, eds (Plenum Press, New York)

Lewis et al., 1978. Assay of ribonucleotide reduction in nucleotide-permeable hamster cells. J. Cell Physiol. 94:287-298.

Lo, 1983. Mol. Cell. Biol. 3:1803-1814

Loke et al, 1989. Characterization of oligonucleotide transport into living cells. PNAS USA 86:3474.

Mann et al., 1988. Ribonucleotide reductase M1 subunit in cellular proliferation, quiescence, and differentiation. J. Cancer Res. 48:5151-5156.

Marzluff and Pandey, 1988. Multiple regulatory steps control histone mRNA concentrations. Trends Biochem. Sci. 13:49-52.

McClarty et al., 1990. Increased ferritin gene expression is associated with increased ribonucleotide reductase gene expression and the establishment of hydroxyurea resistance in mammalian cells. J. Biol. Chem. 265:7539-7547.

Miller et al., 1993. Use of retroviral vectors for gene transfer and expression. Meth. Enzymol. 217:581-599.

Mernaugh and Mernaugh, 1995 "An overview of phage-displayed recombinant antibodies" in Molecular Methods In Plant Pathology (RP Singh and US Singh, eds.; CRC Press Inc., Boca Raton, FL) pp. 359-365.

Morrison, 1991. Suppression of basic fibroblast growth factor expression by antisense oligonucleotides inhibits the growth of transformed human astrocytes. J. Biol. Chem. 266:728.

Nowell, 1986. Mechanisms of tumor progression. Cancer Res. 46:2203-2207.

Pearson and Choi, 1993. Expression of the human β-amyloid precursor protein gene from a yeast artificial chromosome in transgenic mice. Proc. Natl. Scad. Sci. USA 90:10578-82.

Qian et al, 1993. Cloning of a cDNA encoding an RNA binding protein by screening expression libraries using a Northwestern strategy. Biochemistry 212:547-554.

Radhakrishnan et al., 1990. The automated synthesis of sulfur-containing oligodeoxyribonucleotides using 3*H*-1,2-Benzodithiol-3-0ne 1,1 Dioxide as a sulfur-transfer reagent. J. Org. Chem. 55:4693-4699.

Rastinejad et al. 1993. Tumor suppression by RNA from the 3' untranslated region of α-tropomyosin. Cell 75:1107-1117.

Reichard, 1993. From RNA to DNA, why so many ribonucleotide reductases? Science 60:1773-1777.

Ross, 1995. mRNA stability in mammalian cells. Microbiol. Rev. 59:423-450.

Rosolen et al., 1990. Cancer Res. 50:6316.

Rothstein, "Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast" in Methods in Enzymology, Vol. 194, "Guide to Yeast Genetics and Molecular Biology", eds. C. Guthrie and G. Fink, Academic Press, Inc., Chap. 19, pp. 281-301 (1991).

Rowley, 1990. Cytogenetics: Rosetta Stone for understanding cancer. Cancer Res. 50:3816-3825.

Sachs, 1993. Messenger RNA degradation in eukaryotes. Cell 74:413-421.

Saeki et al., 1995. Immunohistochemical detection of ribonucleotide reductase in human breast tumors. Int. J. Oncol. 6:523-529.

Scanlon et al., 1995. Oligonucleotides-mediated modulation of mammalian gene expression. FASEB J. 9:1288.

Schedl et al., "A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice", Nature, Vol. 362, pp. 258-261 (1993).

Schwarz, 1988. Loss of growth factor dependence and conversion of transforming growth factor-β_1 inhibition to stimulation in metastatic H-*ras* transformed murine fibroblasts. Cancer Res. 48:6999-7003.

Shaw et al., 1991. Modified deoxyoligonucleotides stable to exonuclease degradation in serum. Nucleic Acids Res. 19:747-750.

Spearman et al., 1994. Antisense oligodeoxyribonucleotide inhibition of TGF-β_1 gene expression and alterations in the growth and malignant properties of mouse fibrosarcoma cells. Gene 149:25-29.

Spitzer and Eckstein 1988. Inhibition of deoxynucleases by phosphorothioate groups in oligodeoxyribonucleotides. Nucleic Acids Res. 18:11691-11704.

Stokoe et al., 1994. Activation of Raf as a result of recruitment to the plasma membrane. Science 264:1463-1467.

Strauss et al., "Germ line transmission of a yeast artificial chromosome spanning the murine α_1 (I) collagen locus", Science, Vol. 259, pp. 1904-1907 (1993).

Stubbe, 1989. Protein radical involvement in biological catalysis? Annu. Rev. Biochem. 58:257-285.

Taylor et al., 1992. Evidence for synergistic interactions between *ras*, *myc* and a mutant form of p53 in cellular transformation and tumor dissemination. Oncogene 7:1383-1390.

Thelander et al., 1985. Subunit M2 of mammalian ribonucleotide reductase. Characterization of a homogeneous protein isolated from M2-overproducing mouse cells. J. Biol. Chem. 260:2737-2741.

Thelander et al., 1980. Ribonucleotide reductase from calf thymus. Separation of the enzyme into two nonidentical subunits, proteins M1 and M2. J. Biol. Chem. 255:7426-7432.

Thompson et al, 1989. Cell 56:313-321

Tlsty, 1990. Normal diploid human and rodent cells lack a detectable frequency of gene amplification. Proc. Natl. Acad. Sci. USA 87:3132-3136.

Tonin et al., 1987. Chromosomal assignment of amplified genes in hydroxyurea resistant hamster cells. Cytogenet. Cell Genet. 45:102-108.

Uhlenbeck, 1987. Nature 328:596-600

Van der Putten et al, 1985. PNAS USA 82:6148-6152

Wagner et al., 1996. Potent and selective inhibition of gene expression by an antisense heptanucleotide. Nature Biotechnology 14:840-844.

Wagner, 1994. Gene inhibition using antisense oligodeoxynucleotides. Nature 372:333.

Weber, 1983. Biochemical strategy of cancer cells and the design of chemotherapy. Cancer Res. 43:3466-3492.

Whitesell et al., 1991. Episome-generated N-*myc* antisense RNA restricts the differentiation potential of primitive neuroectodermal cell lines. Mol. Cell. Biol. 11:1360.

Wolman, 1983. Karyotypic progression in human tumors. Cancer Metastasis Rev. 2:257-293.

Woolf et al., 1990. The stability, toxicity and effectiveness of unmodified and phosphorothioate antisense oligodeoxynucleotides in *Xenopus* oocytes and embryos. Nucleic Acids Res. 18:1763-1769.

Wright et al., 1987. Altered Expression of Ribonucleotide Reductase and Role of *M2* Gene Amplification in Hydroxyurea-Resistant Hamster, Mouse, Rat, and Human Cell Lines. Somat. Cell Mol. Genet. 13:155-165.

Wright, 1989A. Altered mammalian ribonucleotide reductase from mutant cell lines. Encycl. Pharmacol. Therapeut. 128:89-111.

Wright et al., 1989B. Hydroxyurea and related compounds. *In:* R.S. Gupta (ed.), Drug Resistance in Mammalian Cells, Boca Raton, FL; CRC Press, Inc; 15-27.

Wright et al., 1990A. Regulation and drug resistance mechanisms of mammalian ribonucleotide reductase and the significance to DNA synthesis. Biochem. Cell Biol. 68:1364-1371.

Wright et al., 1990B. DNA amplification is rare in normal human cells. Proc. Natl. Acad. Sci. USA. 87:1791-1795.

Wright et al., 1993. Transforming growth factor β and fibroblast growth factor as promoters of tumor progression to malignancy. Crit. Rev. Oncogen. 4:473-492.

Wright & Anazodo, 1995. Antisense Molecules and Their Potential For The Treatment Of Cancer and AIDS. Cancer J. 8:185-189.

Yakubov et al, 1989. PNAS USA 86:6454.